

Chlorotriptyl Chloride (CTC) Resin as a Reusable Carboxyl Protecting Group¹⁾²⁾

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Abstract

Chlorotriptyl Chloride (CTC) resin is one of the most useful resins for the solid-phase synthesis of C-terminal acid peptides. CTC resin can be used for the preparation of both protected and unprotected peptides. Herein, the use of CTC resin as a reusable mild protecting group of carboxylic acids is introduced. Temporary protection of carboxylic acids in solution is not a straightforward process, but the use of CTC resin is shown to be an alternative to classic solution procedures. Regeneration of the CTC resin after cleavage of the target compounds is feasible, which allows the reuse of the resin. Applications described herein encompass the preparation of noncommercial amino acids as well as complex dipeptides.

1 Introduction

Solid-Phase Synthesis (SPS) is currently the method of choice for peptide synthesis. A stepwise strategy is used

for small and medium peptides, while a convergent strategy is used for large peptides [1–8]. This methodology has also been extended to the synthesis of small molecules and other biomolecules [9–17]. The success of the solid-phase approach is closely tied to the performance of the solid support [18–20]. Chlorotriptyl Chloride (CTC, Barlos) resin is perhaps one of the most useful resins for the SPS of C-terminal peptide acids [21, 22]. It can be used for the preparation of both protected and unprotected peptides. The main advantages of its use are: (i) allows for release of the peptide under mild acidic conditions [1% Trifluoroacetic Acid (TFA), Hexafluoro-2-Propanol (HFIP), TFE] [23]; (ii) minimizes the formation of Diketopiperazine (DKP) [24, 25]; (iii) minimizes racemization during the incorporation of the first amino acid; and (iv) allows for the incorporation of partially protected amino acids through the side chain or the amino function [26, 27].

Although the role that the resin plays in the solid-phase methodology is either permanent (for the preparation of unprotected peptides) or semipermanent (for the preparation of protected peptides to be used in a convergent scheme) [1], CTC resin can also be used as a very acid labile carboxylic temporary protecting group. It is well known that a temporary protection of carboxylic acids in solution is not a straightforward process. For instance, the preparation of *tert*-Butyl (*t*Bu) esters, probably the most common of this kind of protecting group, is a rather diffi-

¹ Preliminary reports of portions of this work were presented at the 19th American Peptide Symposium, June 2005, La Jolla (CA).

² Although the original idea of the Nobel laureate R. Bruce Merrifield (R. B. Merrifield, *J. Am. Chem. Soc.* **1963**, *85*, 2149–2154) was to consider the polymeric support as a permanent protecting group of the C-terminal amino acid for the solid-phase synthesis of peptides, in this manuscript the CTC resin is presented as a temporary protecting group for masking the reactivity of a carboxylic acid in front of the manipulation of other function of the molecule containing the carboxylic acid.

Abbreviations: *Abbreviations.* AcM, acetamidomethyl; Alloc, allyloxycarbonyl; Boc, *tert*-butyloxycarbonyl; CTC, chlorotriptyl chloride; DIEA, *N,N*-diisopropylethylamine; DKP, diketopiperazine; DMF, *N,N*-dimethylformamide; Fmoc, 9-fluorenylmethoxycarbonyl; HPLC, high performance liquid chromatography; MARR, microwave-assisted resin regeneration; MeCN, acetonitrile; MeOH, methanol; MS, mass spectrometry; *p*NZ, *p*-nitrobenzyloxycarbonyl; Ψ Pro, pseudoproline; SEM, scanning electron microscopy; TFA, trifluoroacetic acid; Troc, 2,2,2-trichloroethoxycarbonyl; TFE, trifluoroethanol

cult process that involves the use of a gas and therefore of special equipment. The use of CTC resin overcomes these problems and brings the inherent advantages of the solid-phase approach. First, the use of a large excess of reagents facilitates the completion of the reactions and, second, the work-up only involves filtration and washing to remove the excess of reagents and other soluble by-products.

A main criticism of the solid-phase approach is the cost of the resin. Although recently prices of raw solid-phase materials have decreased due mainly to their high consumption for the production of multikilogram quantities of Active Pharmaceutical Ingredients (APIs) [5], they are still more costly than in solution. Thus, a regeneration process for the used resin would be desirable.³⁾

Herein, the use of CTC resin for the temporary protection of the carboxylic group is described as well as different methods for the regeneration of the resin after cleavage of the target compounds.

2 Results and Discussion

2.1 Regeneration Methods

Three methods are studied to regenerate as well as to activate CTC resin.⁴⁾ While the first method is based on the use of hydrochloric acid as a source of chloride [28], the second uses thionyl chloride [29], and the third uses acetyl chloride in CH_2Cl_2 [30].⁵⁾ The evaluation takes into account the highest loading obtained and the stability of the regenerated resin.

Figure 1 depicts the strategy followed to determine the optimal method for this transformation. Thus in Figure 1, Fmoc-Val-OH (where Fmoc stands for 9-Fluorenylmethoxycarbonyl) in the presence of *N,N*-Diisopropylethylamine (DIEA) was anchored to the initial CTC resin and the loading was calculated by spectrometric quantification of the *N*-fluorenylmethylpiperidine adduct [31]. Then, the protected amino acid was cleaved from the resin with dilute TFA, the resin was regenerated, and Fmoc-Val-OH was incorporated again to measure the loading. As shown in Figure 1, although the acetyl chloride method resulted in slightly lower loading after the second incorporation of the Fmoc-Val-OH, the three methods yielded very similar results (1.12 ± 0.05 mmol/g), which were very close to the initial loading (1.11 mmol/g). However, the thionyl chloride method was the least destructive to the resin in terms

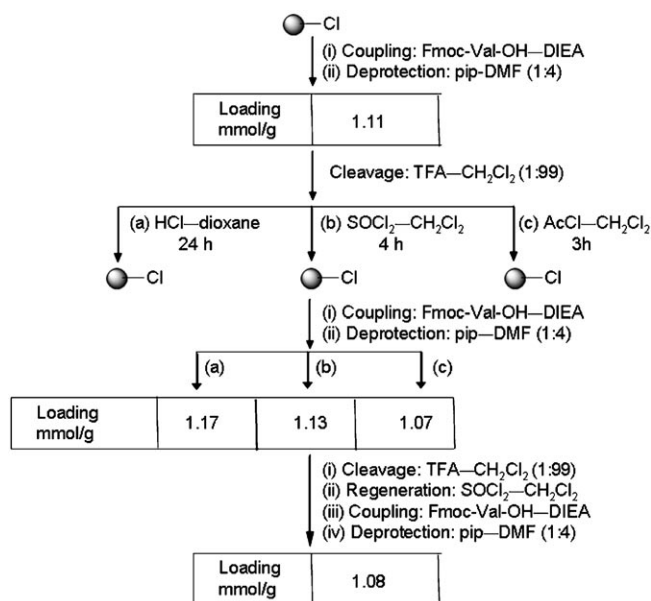


Figure 1. Scheme of Regeneration. The loading is calculated as an average of three experiments.

of deterioration, as resin quality was maintained after regeneration. The most destructive was determined to be hydrochloric acid, followed by the acetyl chloride method. Deterioration was determined by RP-High Performance Liquid Chromatography (HPLC) analysis of CH_2Cl_2 washings performed after the regeneration step. RP-HPLC overlay chromatograms are shown in Figure 2.

Thus, the SOCl_2 method was chosen as the most effective method because it renders higher loading with short reaction times and is the least destructive. Next, a third re-

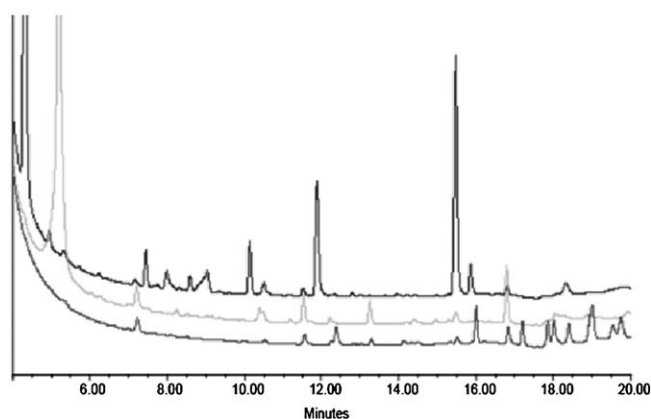


Figure 2. HPLC profile of the residue obtained after evaporation to dryness of the postregeneration CH_2Cl_2 washings (same amount in each for comparison purposes) [Top: HCl method; Middle: AcCl method; Bottom: SOCl_2 method]. RP-HPLC conditions: C_{18} column, linear gradient from 10:0 to 0:10 H_2O (0.045% TFA)—MeCN (0.036% TFA) over 15 min, flow rate of 1.0 mL/min at 220 nm.

³⁾ The term regeneration is preferred over recycling, because the resin after the process has exactly the same properties than initial resin. See Figure 5.

⁴⁾ CTC resins are quite labile during prolonged storage and may be hydrolyzed to the corresponding alcohol. Accordingly, it should be activated prior to use.

⁵⁾ Optimized methods were applied as described by the authors. Thus, reaction times are different in each case.

generation step (Figure 1) was carried out to confirm the efficacy of this method.

2.2 Study of Cross-contamination

The risk of cross-contamination was studied for the chosen thionyl chloride method. Three generations of reactions were carried out with three different amino acids. In the first round, the Fmoc-Val-OH was coupled and cleaved from the resin. Next, the Fmoc-Phe-OH was attached to the regenerated CTC resin and cleaved from the resin. Finally, the Fmoc-Lys(Boc)-OH (where Boc stands for *tert*-Butyloxycarbonyl) was coupled and cleaved from the resin after a further regeneration. The three amino acids were characterized by RP-HPLC (shown in Figure 3). The third

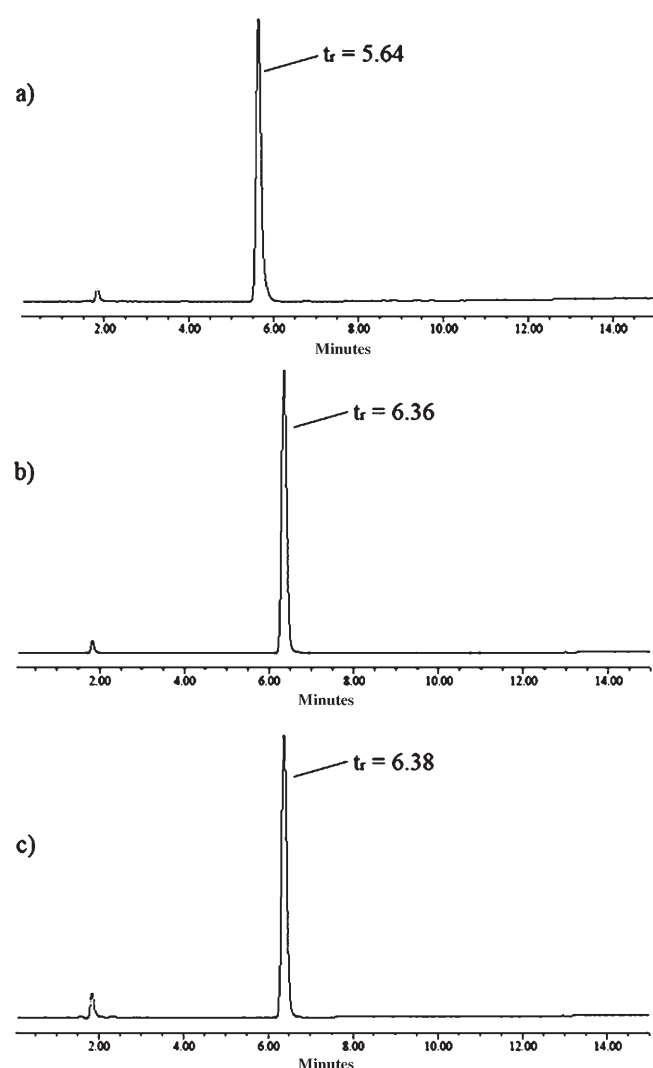


Figure 3. Crosscontamination study. a) Fmoc-Ala-OH, b) Fmoc-Phe-OH, and c) Fmoc-Lys(Boc)-OH. RP-HPLC conditions: C_{18} column, linear gradient from 5:5 to 0:10 H_2O (0.045% TFA)—MeCN (0.036% TFA) over 15 min, flow rate of 1.0 mL/min at 220 nm.

amino acid [Fmoc-Lys(Boc)-OH] was also characterized by HPLC-Mass Spectrometry (MS) due to its similar retention time with the second amino acid (Fmoc-Phe-OH). Only the mass corresponding to Fmoc-Lys(Boc)-OH was detected, while the mass corresponding to Fmoc-Phe-OH was below detection limits. From these results, it can be concluded that the regeneration method takes place without cross-contamination and renders an excellent purity of the final products.

2.3 Regeneration using Microwaves Techniques

Microwave energy may be used as an alternative to conventional thermal heating of chemical reactions. Recently, the microwave technique has been broadly used in organic syntheses due to the fact that reactions take place with faster kinetics and cleaner products are obtained [32]. In particular, the benefits of microwave-assisted SPS has also been established with high-speed couplings and cleavages on Merrifield resin [33], peptide synthesis [34], Suzuki coupling reactions [35], in addition to Knoevenagel [36], and Ugi-type condensation processes [37]. It is especially efficient for high throughput synthesis [38]. To the best of our knowledge, this technique has not been used to regenerate polymeric supports for solid-phase chemistry. A set of experiments based on the initial conditions of the thionyl chloride method and combining time and temperature variables was designed in order to test the Microwave-Assisted Resin Regeneration (MARR). The results shown in Table 1 suggest that there is a rather linear correlation between time/temperature and loading, with temperature being a more important parameter than time. Methods 4 and 5 were found to be the most efficient.

Furthermore, to evaluate the efficacy and the purity rendered by MARR, second and third regeneration treatments were performed after the TFA cleavages using the methods 4 and 5 (see Table 2). Thus, the previous method used for checking the conventional heating strategy (Figures 1 and 3) was used to determine the efficacy of MARR.

For method 4, the first regeneration cycle presents similar purity profiles, but the loading decreases to 0.66 mmol/g resin. This method was ruled out because after the third regeneration cycle the polymer decomposes, rendering a jelly-like appearance. This demonstrates the importance of

Table 1. Influence of temperature and time on MARR. Initial loading as determined above 1.11 ± 0.06 mmol/g. The high loading of experiment #4 can be interpreted by the fact that the T_g of polystyrene is about 100°C and this resin has only 1% cross-linking.

Method	1	2	3	4	5
Time (min)	10	5	12	5	8
T^a ($^\circ\text{C}$)	63	88	70	130	88
Loading (mmol/g resin)	0.45	0.51	0.74	1.82	1.03

Table 2. MARR results for three regeneration cycles. The high loading of experiment #4 can be interpreted by the fact that the T_g of polystyrene is about 100 °C and this resin has only 1% crosslinking.

Method	Time (min)	T^a (°C)	First cycle loading (mmol/g resin)	Second cycle loading (mmol/g resin)	Third cycle loading (mmol/g resin)
4	5	130	1.82	0.66	0.07
5	8	88	1.03	0.74	0.71

utilizing a conservative range of temperature and time, avoiding extreme conditions of either.

For method 5, RP-HPLC profiles are identical to conventional heating, providing a high purity product (Figure 4). However, the loadings were decreasing after the first regeneration (Table 2). In conclusion, MARR gives equal quality with reduced cycle times, although after a second regeneration the loading decreased a little bit.

2.4 MARR Purity by Scanning Electron Microscopy (SEM)

While the above results indicate that MARR method 5 does not affect the morphological characteristics of the resin, this has been further investigated by SEM. SEM is a useful technique for investigating surface structures.

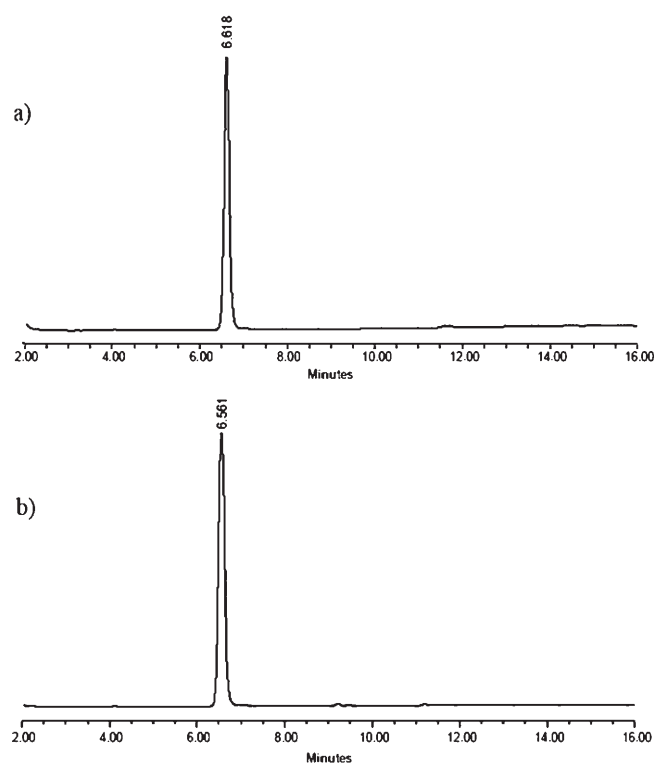


Figure 4. Cross-contamination during MARR process, method 5. RP-HPLC profiles. a) Fmoc-Phe-OH and b) Fmoc-Lys(Boc)-OH. RP-HPLC conditions: C_{18} column, linear gradient from 5:5 to 0:10 H_2O (0.045% TFA)–MeCN (0.036% TFA) over 15 min, flow rate of 1.0 mL/min at 220 nm.

Figure 5 depicts beads analyzed by SEM at different stages of the MARR process. All the images were taken using the lowest possible voltage (1000 V). Figure 5a illustrates the resin directly from commercial flask prior to use. An artifact appears in the center of the bead, suggesting that the beads may be excessively charged even when the lowest voltage is used. Figure 5b shows beads after common TFA treatment [TFA– CH_2Cl_2 (1:99), 5×0.5 min], CH_2Cl_2 washing, and air drying. As shown in Figure 5c, during the MARR process, the resin does not appear to be mechanically affected. This image was taken after method 5, washing with CH_2Cl_2 and drying in the air.

2.5 Regeneration Application

After demonstrating that CTC resin could be an efficient protecting group that offers all solid-phase advantages, such as high yield and purity and short manipulation times, its use in real laboratory applications was investigated. One such application was recently described by our group, the preparation of Biotinylated Alkyl Thiols (BAT) which are widely used for biological applications [39]. Herein, we report several other important and useful applications.

2.5.1 Preparation of Noncommercial Amino Acids

Synthesis of complex peptides may require amino acids protected with different protecting groups. These are often not commercially available and/or difficult to obtain quickly. Thus, it is useful to have available an easy and efficient method for the preparation of the unusual protected amino acids. For example, three different N^α -amino protected amino acids, protected with Allyloxycarbonyl (Alloc), *p*-Nitrobenzyloxycarbonyl (*p*NZ), or Trichloroethoxycarbonyl (Troc) were obtained using the same beads

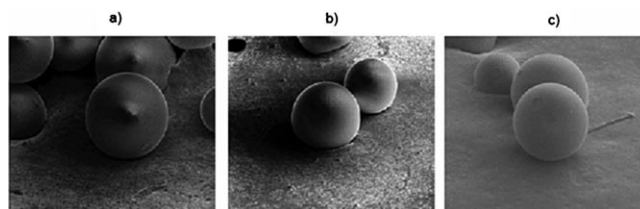


Figure 5. SEM images. a) CTC Resin before being used, b) CTC Resin after TFA treatment, and c) CTC Resin after method 5 of MARR.

repeatedly regenerated (see Figure 6). An advantage of the CTC resin is that the incorporation of the first protected amino acid does not require its use in excess as is required with other solid-phase resins [26]. It also does not require any activation chemistry, avoiding the related costs. To assure a complete protection, 3 equiv. of the

tions were very often quantitative. In addition, the cycle time for the whole process did not exceed 2 h. Compared to solution phase chemistry, this methodology is more effective, cleaner, and faster.

2.5.2 Dipeptides Synthesis

Dipeptides are often required in natural peptide synthesis development. Dipeptide building blocks avoid DKP formation, which might otherwise greatly decrease yield of peptides containing large amounts of *N*-methyl amino acids and depsipeptides with ester linkages forming the peptide chain. To avoid DKP formation, dipeptides with an *N*-methyl amino acid at the C-terminal position are usually required for a direct incorporation with the first amino acid when obtaining a tripeptide[40]. Traditionally, dipeptide synthesis has been performed in solution-phase due to high costs of resins, reagents, and solid-phase capable infrastructure. For one of our ongoing projects[41], the dipeptides Fmoc-Gly-*N*-Me-Cys(Acm)-OH (where Acm stands for Acetamidomethyl) and Alloc-Gly-*N*-Me-Cys(Acm)-OH were needed to avoid DKP in high *N*-methyl containing peptides. These dipeptides have been produced previously in solution with a long synthetic route and with very low yields due to the presence of the *N*-methyl amino acids. Figure 7 shows the scheme for the preparation of these dipeptides in solid phase.⁶) CTC resin facilitates dipeptide synthesis in solid phase with improved yields and a dramatic reduction in the reaction time. CTC resin is being applied for the preparation of the Pseudoproline (Ψ Pro) dipeptides [42–44], which have been widely used in the synthesis of difficult peptide

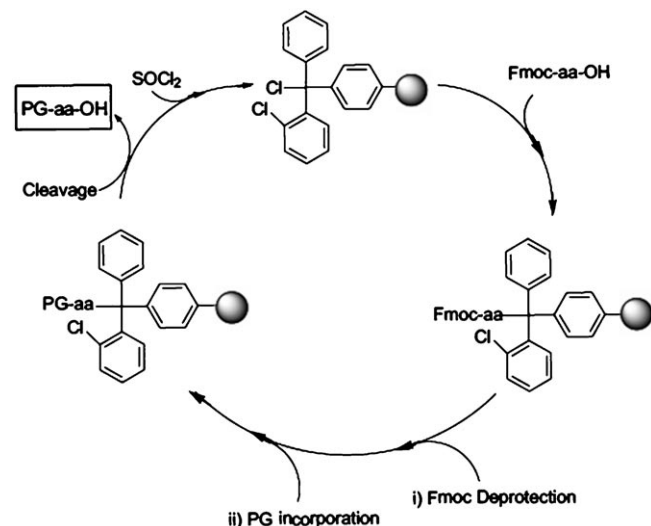


Figure 6. Synthetic scheme used for the preparation of non-commercial amino acids from Fmoc-amino acids. Note that the incorporation of a specialized Protecting Group (PG) can be performed on resin to render the protected peptide.

respective chloroformate protecting reagent were needed. The HPLC-MS profiles reveal the major peak as the target compound and the Nuclear Magnetic Resonance (NMR) spectrums shows clean products. Furthermore, due to the acid lability of the CTC resin, the product recovery was usually stoichiometric and the yields of these transforma-

⁶ These peptides were used without further purification for the solid-phase synthesis of more complex peptides (see Ref. [41]).

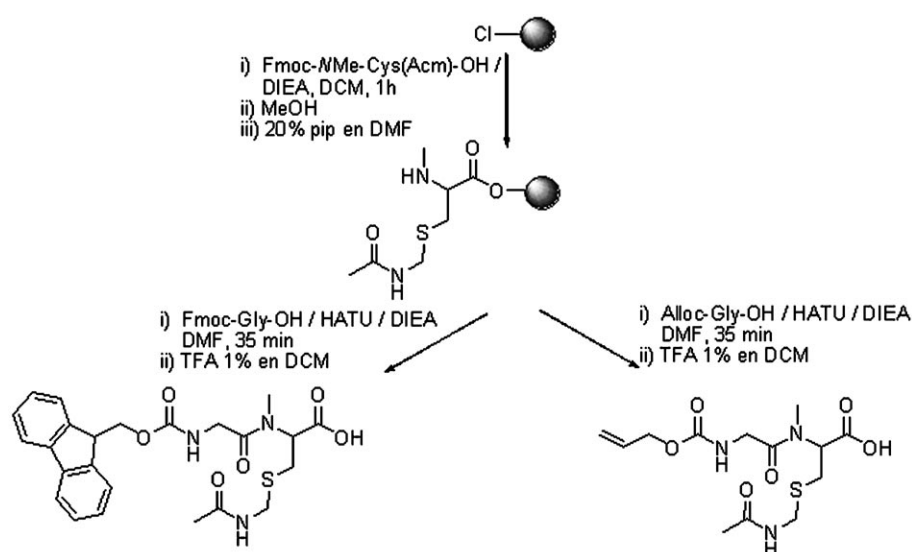


Figure 7. Synthetic scheme for the synthesis of dipeptides on solid phase using regenerated CTC resin.

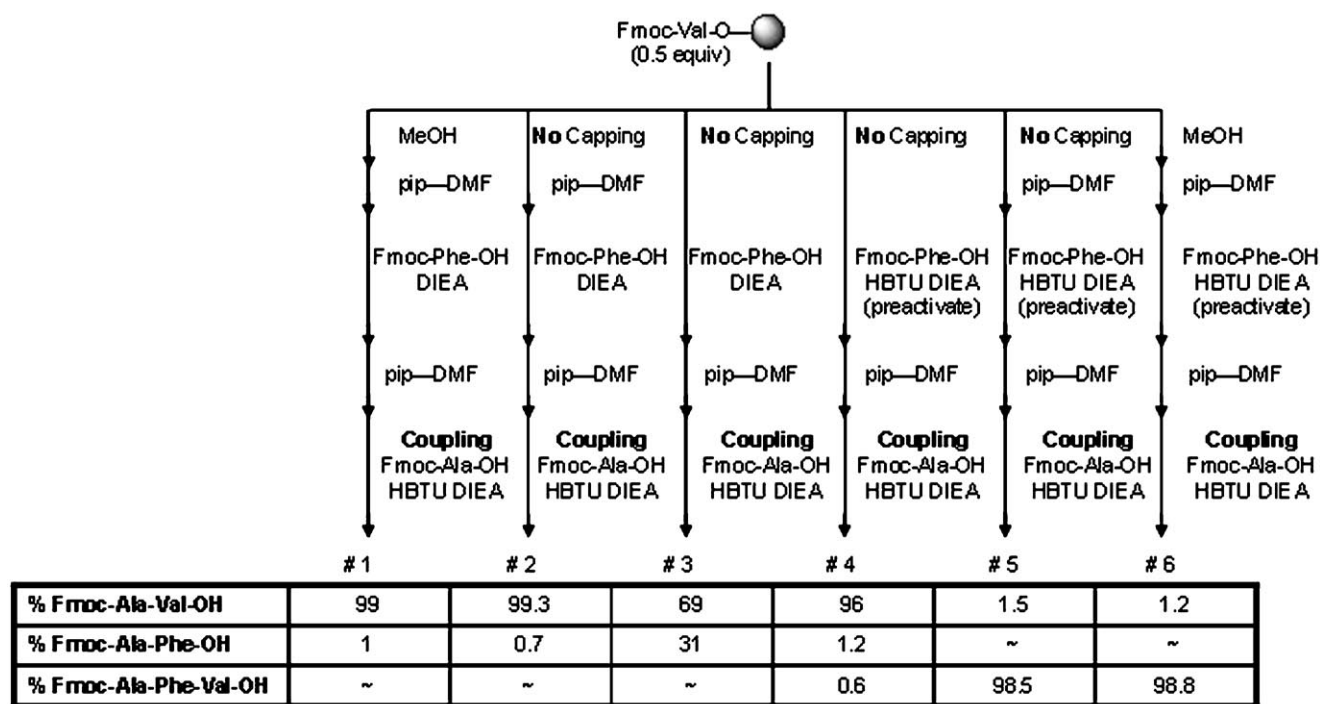


Figure 8. Capping analysis.

sequences because they disrupt the aggregation during peptide assembly [20].

2.6 Study of the “Capping” Step after Incorporation of the First Protected Amino Acid

Usually, after the incorporation of the first amino acid, a Methanol (MeOH) treatment is carried out to cap all of the residual chloride groups contained in the resin [25]. Herein, a possible elimination of this step has been evaluated. This capping step is not tedious in a research laboratory where only a few minutes are spent, but on an industrial scale, the elimination of this step would reduce the cycle time and solvents required, thus reducing the waste. This could also facilitate recycling of both the resin and solvents. Figure 8 shows the method used for this study. A Fmoc-Val-O-CTC resin with approximately 50% of the sites loaded with Fmoc-Val, and therefore with 50% of the sites with unreacted Cl, were divided into six batches: the first two steps of entry #1 show the classic pathway (capping with MeOH and removal of the Fmoc group), then a treatment with Fmoc-Phe-OH and DIEA followed by piperidine treatment to remove the possible Fmoc of the Fmoc-Phe-OH incorporated, and coupling of the Fmoc-Ala-OH. The presence of 1% Fmoc-Ala-Phe-OH could indicate that the capping was not totally achieved and some Fmoc-Phe-OH was incorporated on the CTC-resin. Similarly results were obtained with entry #2, which was similar to #1, but MeOH treatment was not carried out. This means that MeOH capping

is not mandatory. On the other hand, if neither MeOH nor piperidine treatments were carried out, the treatment with Fmoc-Phe-OH + DIEA (#3) resulted in a 31% incorporation of the Phe residue. This incorporation was minimized when Fmoc-Phe-OH was incorporated with preactivation (#4). This indicates that no free DIEA salt of Fmoc-Phe-OH was present. Entries #5 and #6 reinforced the results of entry #2, which showed that piperidine was able to cap the free Cl.

3 Conclusions

CTC has been shown to be a convenient and efficient temporary protecting group, which can be used for the rapid preparation of protected amino acids or small peptides. After the desired compound is obtained, CTC is repeatedly regenerated and reused. Furthermore, the use of microwave technique has allowed a faster and cleaner strategy for the resin regeneration step when compared with the thermal conditions. The possibility of the regeneration of CTC resin enables it to be considered as a chemical tool for daily laboratory work instead of just a consumable reagent. The capping study revealed that treatment with MeOH is not necessary to remove the free Cl because the same action is performed by piperidine. Moreover, incorporation of the second residue with preactivation further minimized its incorporation onto possible free Cl groups.

4 Experimental Section

4.1 Material and General Methods

4.1.1 Materials and Equipment

CTC resin was obtained from Rohm & Haas (Spring House, PA). Protected L-amino acids were obtained from Luxembourg Industries (Tel-Aviv, Israel), Neosystem (Strasbourg, France), Calbiochem-Novabiochem AG (Laüfelfingen, Switzerland), Bachem AG (Bubendorf, Switzerland), and Iris BioTech GmbH (Ciudad, Germany). Ac₂O was supplied by Calbiochem-Novabiochem AG. TFA was obtained from Fluka Chemika (Buchs, Switzerland) and 1-[bis(dimethylamino)methylene]-1H-benzotriazolium hexafluorophosphate 3-oxide (HBTU) from Luxembourg Industries. Piperidine was supplied by SDS (Peypin, France). DIEA was obtained from Merck Schuchardt (Hohenbrunn, Germany). Solvents for peptide synthesis and Reverse-Phase (RP)-HPLC were obtained from Scharlau (Barcelona, Spain) and SDS. CH₂Cl₂ is passed by silica column before use. Other chemicals used in the present work were obtained from Aldrich (Milwaukee, WI, USA) and were of the highest purity available. RP-HPLC was performed on a Waters Alliance 2695 chromatographic system (Waters, MA, USA) with a PDA 995 detector and a reverse-phase Symmetry C₁₈ column (4.6 Ø150 mm, 5 µm). Linear gradients of 0.045% TFA in H₂O and 0.036% TFA in Acetonitrile (MeCN) were run at the flow rate of 1.0 mL/min. HPLC-MS was performed on a Waters Alliance 2796 with UV/Vis detector 2487 and an ESI-MS Micromass ZQ chromatographic system (Waters), using a reverse-phase Symmetry 300 C₁₈ column (3.9 Ø150 mm, 5 µm), 0.1% formic acid in H₂O, and 0.07% formic acid in MeCN were used as eluents. Mass spectra were recorded on a Matrix-Assisted Laser Desorption Ionization (MALDI) Voyager DE RP Time-of-Flight (TOF) spectrometer (PE Biosystems, Foster City, CA, USA). Regeneration steps take place in the LabMate system (Advanced ChemTech, Louisville, KE, USA), which permits several reactions in parallel at different temperatures. ¹H NMR spectra were obtained on a Mercury 400 (400 MHz) spectrometer (High Field NMR Unit, Barcelona Science Park) in CDCl₃ unless stated otherwise. Chemical shifts are reported in ppm (δ units) downfield from internal Tetramethylsilane (TMS). Microwave used in MARR reactions is "Discover CEM".

4.1.2 General Procedures

Manual Synthesis: Manual solid-phase amino acid incorporation and other solid-phase manipulations were carried out in polypropylene syringes fitted with a polyethylene porous disk. Solvents and soluble reagents were removed by suction. Fmoc removal was carried out with piperidine-*N,N*-Dimethylformamide (DMF) (1:4) (1 × 1 min,

2 × 10 min). Washings between deprotection, coupling, and subsequent deprotection steps were carried out with DMF (3 × 1 min) and CH₂Cl₂ (3 × 1 min) using 10 mL of solvent/g of resin each time. Cleavage step was carried out using TFA-CH₂Cl₂ (1:99) (5 × 0.5 min) followed by several washings: CH₂Cl₂ (2 × 1 min), DMF (2 × 1 min), DMF-H₂O (2 × 1 min), DMF (2 × 1 min), and CH₂Cl₂ (2 × 1 min). The traces of TFA that remains on the product was removed by coevaporation with toluene. Alternatively, noncarboxylic acids such as HFIP or TFE methods can be used to cleave the building blocks from CTC resin.

Loading Calculation: Deprotection with piperidine gives the fulvene-piperidine adduct which can be determined by quantitative spectrophotometric at 290 nm (E₂₈₀ = 5800).

Storage of the Resin: As it is indicated in Section 2, the resin after regeneration presented a higher loading than prior use (from commercial flask). This phenomenon could be due to partial destruction of the commercially available CTC resin during the transport and storage. Thus, the CTC resin should be carefully stored at 4 °C.

Regeneration Methods: Each of the three methods was carried out in triplicate while using the same conditions as follows: nine syringes with 100 mg of CTC each one were washed with DMF (3 × 1 min), CH₂Cl₂ (3 × 1 min), DMF (3 × 1 min), and CH₂Cl₂ (3 × 1 min); Fmoc-Val-OH (4 equiv.)⁷ and DIEA (20 equiv.) were sequentially added to the resin and the mixture was allowed to react for 90 min. Next, a capping step with MeOH (60 µL) was carried out, following the Fmoc-removal. The piperidine-DMF and consecutive DMF washings were collected in order to calculate the loading. The cleavage was made using TFA-CH₂Cl₂ (1:99) (5 × 0.5 min), when the resin adopted a red coloration. Finally, several washings were realized: CH₂Cl₂ (2 × 1 min), DMF (2 × 1 min), DMF-H₂O (2 × 1 min), DMF (2 × 1 min), CH₂Cl₂ (2 × 1 min), and the resin, that had recovered the initial pale yellow coloration, was air dried.

Three regeneration methods, using 100 mg of CTC resin, were tested: (i) 4 M HCl-dioxane (2 mL) was added and the mixture stirred at room temperature for 24 h and (ii) resin was swelled in CH₂Cl₂ (1 mL) for 10 min. Next, SOCl₂ (25.8 µL) was added dropwise and the mixture turned darker. Consecutively, DMF (5% v/v SOCl₂) was added and the dark color lightened slightly. The mixture was stirred under Ar atmosphere for 4 h at 38 °C. (iii) 10% AcCl-CH₂Cl₂ (2 mL) was added and the mixture stirred at room temperature for 3 h. Resins were washed with DMF (3 × 1 min) and CH₂Cl₂ (3 × 1 min), and the washings were collected to analyze the bead destruction by RP-HPLC.

⁷ Four equivalents of Fmoc-Val-OH were added in order to determine the maximum loading from the resin.

4.2 Synthesis of Noncommercial Amino Acid Derivatives

Amino acid 1: Fmoc-Val-OH (2 equiv.) was coupled to CTC (500 mg) using DIEA (10 equiv.) as base. After capping with MeOH, the Fmoc group was removed by piperidine, next the *p*NZ group was incorporated in the amine group by two treatments with *p*NZ-Cl (3 equiv.) and DIEA (15 equiv.) in 30 min. Total reaction was followed by Kaiser test [45]. After cleavage, the desired amino acid was obtained in a 61% yield (yield no optimized) as solid powder and characterized by HPLC-MS (t_R 10.02, m/z calcd for $C_{13}H_{16}N_2O_6$, 296.1; found 295.2 $[M-H]^-$) and 1H NMR ($CDCl_3$, 400 MHz): 1.01 (d, 3 H, $-CH_3$), 1.03 (d, 3 H, $-CH_3$), 2.25 (m, 1 H, $-CH^{\beta}$), 4.35 (dd, $J=8.5$, 5.9 Hz, 1 H, $-CH^{\alpha}$), 5.22 (s, 2 H, $-CH_2$ *p*NZ), 5.35 (d, 1 H, $-NH$), 7.51 (d, $J=8.2$ Hz, 2 H, CH_{ar}), 8.22 (d, $J=8.8$ Hz, 2 H, CH_{ar}) ppm.

Amino acid 2: Once the first protected amino acid was obtained, the resin was regenerated by $SOCl_2$ treatment and the Fmoc-Phe-OH was coupled as before and again Fmoc group was removed. Then, allyl chloroformate (3 equiv.) was incorporated with DIEA (15 equiv.) for 50 min in order to obtain Alloc-Phe-OH. The second amino acid was cleaved from the resin with quantitative yield and characterized by HPLC-MS (t_R 9.57, m/z calcd for $C_{13}H_{15}NO_4$, 249.1; found 250.1 $[M+H]^+$) and 1H NMR ($CDCl_3$, 400 MHz): 3.16 (m, 2 H, $-CH^{\beta}_2$), 4.56 (d, $J=5.2$ Hz, 2 H, $-OCH_2$ Alloc), 4.68 (m, 1 H, $-CH^{\alpha}$), 5.13 (d, $J=8.0$ Hz, 1 H, $-NH$), 5.21 (d, $J=10.8$ Hz, 1 H, $HH'=CH_2$ Alloc), 5.28 (d, $J=17.2$ Hz, 1 H, $HH'=CH_2$ Alloc), 5.88 (m, 1 H, $=CH$ Alloc), 7.16–7.34 (m, 5 H, CH_{ar}) ppm.

Amino acid 3: Following the cycle, the resin was again regenerated and Fmoc-Ala-OH was incorporated as above. After deprotection, the Troc group was incorporated by Troc-chloroformate (2 equiv.) using DIEA (10 equiv.) in two treatments of 45 min. After cleavage, Troc-Ala-OH was obtained with a yield of 49% (yield no optimized) and was characterized by HPLC-MS (t_R 9.15, m/z calcd for $C_6H_8Cl_3NO_4$, 262.9; found 261.9 $[M-H]^-$) and 1H NMR ($CDCl_3$, 400 MHz): 1.53 (d, $J=7.2$ Hz, 3 H, $-CH_3$), 4.45 (m, 1 H, CH^{α}), 4.74 (m, 2 H, $-OCH_2$ Troc), 7.26 (s, 1 H, $-NH$) ppm.

4.3 *N*-Methyl Amino Acids Containing Dipeptides

Dipeptide synthesis started by a limited anchoring of Fmoc-NMe-Cys(Acm)-OH onto the CTC resin (0.7 mmol of protected amino acid *per* g of resin) during 1 h. The Fmoc group was removed with piperidine-DMF (1:4; 1 × 1 min, 3 × 5 min, 1 × 10 min) and Fmoc-Gly-OH coupling was performed with the 1-[bis(dimethylamino)methylene]-1H-1,2,3-triazolo-[4,5-b]pyridinium hexafluorophosphate 3-oxide (HATU), with DIEA, which assured a complete acylation of the *N*-methylamino acids. The coupling reaction was tested by the De Clercq test [46]. Coupling was achieved in 35 min. In the other dipeptide,

Alloc-Gly-OH was coupled directly to *N*-methyl free group as is described above. In this case was not possible interchange of Fmoc group by Alloc group was not possible because NMe-Cys(Acm) in C-terminal was susceptible to β -elimination by piperidine treatments. Dipeptides were cleaved from CTC with mild acidic conditions 1% TFA in CH_2Cl_2 (5 × 30 s). Solvent was removed under pressure and Fmoc-Gly-NMe-Cys(Acm)-OH was obtained in an 87% yield and characterized by RP-HPLC (7:3-0:10 in 15 min, t_R 6.6 min, 97%) and MS (m/z calcd for $C_{24}H_{27}N_3O_6$, 485.2; found 486.4 $[M+H]^+$). The compound, Alloc-Gly-NMe-Cys(Acm)-OH was obtained in a 75% yield and characterized by RP-HPLC (10:0-0:10 in 15 min, t_R 6.5 min, 90%) and MS (m/z calcd for $C_{13}H_{21}N_3O_6S$, 347.1; found 348.3 $[M+H]^+$). Both compounds were obtained in less than 3 h.

4.4 MeOH Capping Study

Six studies were carried out simultaneously to evaluate the capping ability of MeOH and piperidine (see Figure 8). These studies were performed as follows: CTC resin (600 mg) was washed with CH_2Cl_2 (3 × 1 min), DMF (3 × 1 min) and CH_2Cl_2 (3 × 1 min). Subsequently, coupling with Fmoc-Val-OH (0.5 equiv.) and DIEA (20 equiv.) was carried out. Next, the resin was split in six batches. These samples were then treated with different treatment/coupling steps. MeOH treatment: MeOH (200 μ L) was added to the resin mixture; piperidine treatment was carried out as before; Fmoc-Phe-OH (5 equiv.) and DIEA (20 equiv.) in DMF; Fmoc-Phe-OH and Fmoc-Ala-OH (5 equiv.) were preactivated by HBTU (4.5 equiv.) and DIEA (20 equiv.) in DMF.

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